PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau

AD

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : C12N 7/00, 9/24, 5/00 C12N 15/00, C07H 21/00 A61K 37/54 — A1

(11) International Publication Number: WO 89/ 05850

(43) International Publication Date: 29 June 1989 (29.06.89)

(21) International Application Number: PCT/US88/04314

(22) International Filing Date: 30 November 1988 (30.11.88)

(31) Priority Application Number: 137,796

(32) Priority Date: 23 December 1987 (23.12.87)

(33) Priority Country: US

(71) Applicant: THE UNITED STATES OF AMERICA, as represented by THE SECRETARY, U.S. DEPARTMENT OF COMMERCE [US/US]; 5285 Port Royal Road, Springfield, VA 22161 (US).

(72) Inventors: GINNS, Edward, I. ; 8617 Rayburn Road, Bethesda, MD 20817 (US). MARTIN, Brian ; 5608 Lone Oak Drive, Bethesda, MD 20184 (US). MAYSACK, Kara, A. ; 2601 Spencer Road, Chevy Chase, MD 20815 (US). ELIASON, William, K. : 2258 Gunsmith Square, Reston, VA 22091 (US). LaMARCA, Mary, E. ; 5127 Baltimore Avenue, Bethesda, MD 20816 (US).

(74) Agents: STERN, Marvin, R. et al.; Holman & Stern, 2401 Fifteenth Street, N.W., Washington, DC 20009 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: CLONED DNA FOR SYNTHESIZING UNIQUE GLUCOCEREBROSIDASE

(57) Abstract

A cloned cDNA for synthesis of unique glucocerebrosidase is provided. The enzyme thus produced is different from heretofore known similar enzymes.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
DE	Germany, Federal Republic of	LU	Luxembourg	TG	Togo
DK	Denmark	MC	Monaco	US	United States of America
FI	Finland	MG	Madagascar		

CLONED DNA FOR SYNTHESIZING UNIQUE GLUCOCEREBROSIDASE

BACKGROUND OF THE INVENTION

Technical Field:

The present invention is related to the construction of an expression vector for the synthesis of a recombinant enzyme. More particularly, the present invention is related to the large scale production of glucocerebrosidase by infecting invertebrate cells with a recombinant baculovirus containing the complete cDNA sequence for encoding glucocerebrosidase.

State of the Art:

Mutation or deficiency of the lysosomal glycoprotein glucocerebrosidase (EC 3.2.1.45, β-D-glucosyl-N-acylsphingosine glycohydrolase) results in Gaucher's disease. It is estimated that there are about 20,000 cases of this genetic disease in the U.S. alone.

Published methods for producing large quantities of the active human enzyme involve purification of the protein from large amounts of human tissue, such as placenta. It should be noted, however, that the placental glucocerebrosidase has carbohydrate structure different than that of the enzyme found in human liver, spleen, brain or macrophages.

Construction of a cDNA clone containing the entire human glucocerebrosidase coding region has been known (Sorge, et al., Proc. Natl. Acad. Sci. USA, 82:7289-7293, 1985). However, as it will become clear vide infra, both the cDNA clone of the present invention and the enzyme synthesized therefrom, are qualitatively different from the similar prior art entities.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide an active clone of human cDNA containing the complete coding region for the lysosomal glycoprotein glucocerebrosidase (GCS), preferably introduced into the genome of Autographa californica nuclear polyhedrosis virus downstream to the polyhedrin promoter.

It is a further object of the present invention to provide synthetic, isolated and substantially pure recombinant GCS in which the carbohydrate moiety in the glycoprotein structure is different from the human placental GCS.

It is another object of the present invention to provide a method for large scale production of recombinant GCS by infecting Spodoptera frugiperda cells with the recombinant vector of the present invention.

It is a still further object of the present invention to provide a method for treating Gaucher's disease comprising administering to a subject inflicted with Gaucher's disease, therapeutic amount of the recombinant GCS to alleviate the disease condition.

Other objects and advantages of the present invention will become apparent from the following Detailed Description of the Invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

Fig. 1 shows DNA sequence of a human glucocerebrosidase cDNA used for the construction of the baculovirus derived vector, pAC373/GC. In addition to the nucleotide sequence, the amino acids encoded by the coding sequence of the cDNA for human lysosomal glucocerebrosidase is also shown;

Fig. 2 shows schematic construction of baculovirus derived vector containing cDNA for human glucocerebrosidase. The cDNA for human glucocerebrosidase containing the sequence shown in Figure 1 was blunted and then ligated into the SmaI site of a pUC vector (for instance pUC19) yielding pUC19/GC with the cDNA for human glucocerebrosidase lying between unique EcoRI and XbaI sites. The human glucocerebrosidase cDNA could be excised with EcoRI and XbaI, blunted, and ligated in a blunted BamHI site in the baculovirus derived vector, pAC373/GC. This baculovirus vector construct, pAC373/GC, contains human glucocerebrosidase cDNA downstream from the polyhedrin promotor in a 5' to 3' orientation;

Fig. 3 shows comparative data of pH profiles of human placental glucocerebrosidase and the recombinant enzyme produced by using the baculovirus expression system. The recombinantly produced human glucocerebrosidase in both the cell pellet and the culture supernatant has a broad range of pH activity

(between pH 3.5 and pH 8.0) with pH optima at approximately pH 4.5 and pH 5.5. The human placental enzyme has a broad range of pH activity (between pH 3.5 and pH 8.0) with pH optima at approximately pH 5.0 and pH 6.0;

Fig. 4 presents comparative results by Western blot analysis of human placental glucocerebrosidase and the recombinant human glucocerebrosidase produced in the eucaryotic SF9 cells. Sample preparation, electrophoresis and Western blot analysis were performed as described in the text. Molecular weight size markers (Mr $X10^{-3}$) were phosphorylase b, albumin, ovalbumin, and carbonic anhydrase. P, placental enzyme; M, media glucocerebrosidase; and C, cell-associated glucocerebrosidase. Western blot from untreated placental enzyme and recombinantly produced protein are shown in lanes P1 and, M4 and C7, respectively. Cross reactive material (CRM) from endoglycosidase-H and N-glycanase digested samples are shown in lanes P2, M5, C8, and P3, M6, C9, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved by a cDNA clone containing the complete coding sequence for human lysosomal glucocerebrosidase as shown in Figure 1, said clone having been inserted into the genome of Autographa californica nuclear polyhedrosis virus downstream to the polyhedrin promoter as shown schematically in Figure 2.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art

to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

The term "substantially pure" as defined herein means as pure as can be obtained by standard purification techniques known to one of ordinary skill in the art.

MATERIALS AND METHODS

Materials: Restriction endonucleases and recombinant enzymes were obtained from either Life Science Technologies or New England Biolabs. Concanavalin A-Sepharose was obtained from Pharmacia. Octyl-Agarose and Decyl-Agarose were purchased from ICN Biomedicals, Inc. Polyvinylidene difluoride (PVDF) membranes, 0.45 μm pore size, were obtained from Millipore Corp. Sequencer chemicals and solvents for on-line PTH analysis were purchased from Applied Biosystems Inc. Endoglycosidase H was from Miles Scientific while N-Glycanase was purchased from Genzyme Corp.

Construction of recombinant Baculoviruses: Spodoptera frugiperda SF9 cells, plasmid pAc373, and wild-type AcNPV strain E2 were obtained from Max Summers, Texas A&M University. The SF9 cells were maintained in culture at 28°C using TNM-FH media (GIBCO) (Hink, Nature, 226:466, 1970). The cDNA for human glucocerebrosidase was obtained from plasmid pUC19/GC, a derivative of an Okayama-Berg clone from a SV40 transformed human fibroblast cDNA library (Okayama, et

al., Mol. Cell Biol., 3:280, 1983). This cDNA contained 5'and 3' untranslated sequences as well as the complete coding region for glucocerebrosidase. As shown in Figure 2, pAc373/GC was generated by ligation of the blunted EcoRI-XbaI fragment from pUC19/GC into the blunted unique BamHI site of pAC373. Correct orientation of the inserted glucocerebrosidase cDNA was determined by restriction endonuclease analysis.

Recombinant baculovirus containing the human glucocerebrosidase coding sequence under transcriptional control of the polyhedrin promoter was produced by cotransfection of wild-type virus, AcNPV, with plasmid pAc373/GC into SF9 cells as described by Summers, et al., (Tex. Agric. Exp. Stn. Bull. No. 1555, 1987). Five to six days after cotransfection, virus was harvested from the culture supernatant and used to inoculate new monolayers of SF9 cells in petri dishes that were subsequently overlaid with 1% low melting agarose containing TNM-FH medium. Seventy-two hours later the agarose overlay was removed and stored at 4°C, and the cell monolayer was blotted onto a nitrocellulose disk (BA85, Schleicher & Schuell). The disk was hybridized to the random primed, ^{32}P labelled EcoRI-XbaI glucocerebrosidase cDNA fragment from pUC19/GC. Areas on the agarose overlay corresponding to points on the nitrocellulose disk showing hybridization signal were excised and placed in one milliliter of TNM-FH medium. This virus was used for infection of SF9 monolayer cultures and an additional 5 cycles of infection-hybridization were carried out during the plaque purification procedure.

A deposit of PAc373/GC has been made at the ATCC, Rockville, Maryland on November 30, 1987 under the

accession number 40393. The deposit shall be viably maintained, replacing if it became non-viable, for a period of 30 years from the date of the deposit, or for 5 years from the last date of request for a sample of the deposit, whichever is longer, and made available to the public without restriction in accordance with the provisions of the law. The Commissioner of Patents and Trademarks, upon request, shall have access to the deposit.

Enzyme purification: Recombinantly produced glucocerebrosidase was isolated using a modification of the procedure described by Furbish, et al., (Proc. Natl. Acad. Sci. USA, 74:3560, 1977). Cell culture supernatants were precipitated with 195 gm/liter ammonium sulfate. SF9 cell pellets containing the recombinantly produced glucocerebrosidase were extracted into 20 milliliters of sodium phosphate buffer, pH 6.5, containing 150 mM NaCl and 0.1% Triton X-100, followed by sonication twice at 50W for 10 seconds. After precipitation with ammonium sulfate (195 gm/liter) the resuspended pellets were extracted with n-butanol, but ultrafiltration using a YM30 membrane (Amicon) replaced dialysis. After decyl-agarose and octyl-agarose hydrophobic interaction chromatrography at room temperature (about 22°-25°C), the fractions containing glucocerebrosidase activity were pooled, and the ethylene glycol concentration reduced using an Amicon ultrafiltration cell fitted with a YM30 membrane.

Substantially pure enzyme is then obtained following standard conventional purification techniques well known in the art.

Carbohydrate characterization. Endoglycosidase-H was dissolved in 100 mM sodium acetate, pH 6.0, at a

final concentration of 10 units/ml. N-glycanase was supplied as a 250 unit/ml suspension in 50% glycerol. Either human placental enzyme or fifty microliter aliquot of decyl-agarose fraction containing glucocerebrosidase activity were adjusted to 0.5% $NaDodSo_4$/1M β-mercaptoethanol and boiled for two minutes. The samples were then diluted with appropriate buffer to either 200 mM sodium acetate, pH 6.0 (for endoglycosidase-H) or 200 mM sodium phosphate, pH 8.5 (for N-glycanase) to a final composition of 0.1% SDS, 0.7% NP-40, and 0.02M β-mercaptoethanol. The samples were again boiled for 1 min and then either endoglycosidase-H or N-glycanase added to final concentrations of 50 mu/ml or 20 Ul/ml, respectively. Digestions were for about 16 hours at 37°C. Carboxypeptidase Y was used as a control for both deglycosylation reactions.

Western blot analysis: $NaDodSO_4$ polyacrylamide gel electrophoresis and Western blot analysis were performed as described by Ginns, et al., (Proc. Natl. Acad. Sci. USA, 79:5607, 1982).

Amino acid sequence analysis: Samples used for amino acid sequence analysis were electrophoretically fractionated on $NaDodSO_4$ polyacrylamide gels as described above and then transferred to PVDF membranes as described by Matsudaira (J.B.C., 262:10035, 1987). Typically, after electrophoresis the gel was incubated in transfer buffer (0.01M CAPS, 10% methanol, pH 11.0) for 10 minutes prior to transblotting (50 ma for 4 hours). The gel was then washed with HPLC grade water for 5 minutes, stained with 0.1% Coomassie Blue R250 (in 50% methanol) for 5 minutes, and finally destained for 10 minutes with 50% methanol-10% acetic acid. The PVDF

membrane was again washed with HPLC grade water, dried under a stream of nitrogen and stored in a sealing bag at -20°C until used for amino acid sequencing.

Amino acid sequence analysis was accomplished using an Applied Biosystems Model 470A gas-phase sequencer equipped with a Model 120A on-line PTH-amino acid analyzer. The program 03R PTH was used directly for sequencing without pretreatment of the membrane strip with polybrene. An approximately 2 x 8 mm piece of PVDF membrane containing the protein band of interest was excised, centered on the teflon seal, and placed in the cartridge block of the sequencer. Multiple strips of the PVDF membrane could be stacked in this manner, thus increasing the amount of protein available for sequencing. The initial and repetitive yields for sequencing recombinant glucocerebrosidase were calculated by comparison with the yields obtained after 100 picomoles of human placenta glucocerebrosidase were electrophoresed, transblotted to PVDF and subjected to ten cycles of amino acid sequence (Table 1).

Table 1 compares the N-terminal amino acid sequence of mature human placental glucocerebrosidase to N-terminal amino acid sequence of recombinant human glucocerebrosidase using the methods described in the text. The N-terminal amino acids determined by direct chemical sequencing of the mature human placental and recombinant glucocerebrosidase are identical indicating that the signal sequence in the recombinantly produced enzymes are correctly processed. The blank in amino acid position 4 of the recombinant enzyme sequence is consistent with cysteine because cysteine was only identified in the placental enzyme following reduction and alkylation of the protein. The vertical arrow above

the human cDNA sequence indicates the site of peptidase cleavage of the signal sequence.

TABLE 1

```
ATG GCT GGC.....TCA GGT GCC CGC CCC TGC ATC CCT AAA AGC TTC GGC   :cDNA
 M  A  G       S  G  A  R  P  C  I  P  K  S  F  G

                A  R  P  C  I  P  K  S  F  G   :placental enzyme

                A  R  P  -  I  P  K  S  F  G   ::culture media

                A  R  P  -  I  P  K  S  F  G   :SF9 cell pellet
```

Glucocerebrosidase assays: For pH profile and inhibition studies, glucocerebrosidase activity was measured using 100 mM potassium phosphate buffer containing 0.15% Triton X-100, 2.5 µl of β-D-[1-^{14}C] glucocerebroside (7.5 mg/ml in sodium taurocholate at 50 mg/ml), and the sample in the total volume of 200 ul. Preincubations with conduritol-B-epoxide were for 30 min at 37°C. For Km determination, β-glucosidase activity was assayed at pH 5.9 using the artificial substrate 4-methylumbellifery-β-D-glucopyranoside (4MUGP) in 100 mM potassium phosphate buffer containing 0.15% Triton X-100 and 0.125% sodium taurocholate. Purification of recombinant glucocerebrosidase was also monitored using 4MUGP.

Figures 1-4 and Table 1 show the comparative results demonstrating the distinctive nature and properties of the cDNA clone and GCS of the present invention relative to the other known similar clones and enzymes, particularly comparing Sorge et al clone and placenta enzyme.

The distinctive properties are listed below:

(1) The human cDNA of the present invention for glucocerebrosidase differs in both nucleotide sequence and translated amino acid sequence from that of Sroge, et al., (PNAS, 1985, and Correction PNAS, 1986). Specifically, the cDNA of the present invention encodes for Leu (at 489) and Arg (at 514) while that of Sroge, et al., encodes Pro and His at position 489 and 514, respectively. In addition, this cDNA sequence differs in three nucleotides from that reported by Tsuji, et al., (J.B.C., 261:50, 1986).

(2) The high level baculovirus expression system differs from other expression systems as should be known to those familar with the subject. For example, the proteins expressed using bacterial hosts do not have the carbohydrate moieties that are added by eukaryotic hosts. Transient expresion systems utilizing COS cells or L cells produce only about 200,000 Units glucocerebrosidase/liter (Choudary, et al., 1986) while the Baculovirus expression system produces over 2,400,000 units glucocerebrosidase/liter after three days of culture. Similarly, enzyme produced in heterologous cells following retroviral gene transfer produces approximately 200,000 units glucocerebrosidase/liter (Choudary, et al., 1986, Cold Spring Harbor Symposia, Vol LI: 1047).

(3) The purification of human glucocerebrosidase from large amounts of human placenta must take into account the risk of the possible presence of infectious agents (such as but not limited to AIDS virus and hepatitis virus). The recombinantly produced glucocerebrosidase is not associated with these potential complications.

(4) The carbohydrate structure of glucocerebrosidase isolated from human placenta is different from that of recombinantly produced glucocerebrosidase by the baculovirus system (see Figure 4).

(5) Several biochemical parameters of the human placental enzyme are different than that of the recombinant glucocerebrosidase produced by employing the baculovirus expression system:

1) The human placental enzyme on Western blot analysis showed a major band of cross reactive material (CRM) at 65 kDa (see Figure 4), while the recombinantly produced enzyme has multile CRM forms between 67 and 52 kDa. However, upon enzymatic removal of carbohydrate, both the recombinantly produced and placental enzyme has a single major CRM form at 52 kDa.

2) The recombinant enzyme was active between pH 3.5 and pH 8.0 with pH optima at pH 4.5 and pH 5.5. The human placental enzyme was active between pH 3.5 and pH 8.0 with pH optima at pH 5.0 and pH 6.0 (see Figure 3).

3) The recombinantly produced enzyme in the media and cell pellet have Km's of 3.3 mM and 3.6 mM. respectively. the Km for the placental enzyme is reported to be 8 mM (Basu, et al., J.B.C., 259:1714, 1984).

It is clear from the above that the recombinantly produced GCS of the present invention is a quantitatively different protein than any other heretofore known entity.

Since the carbohydrate pattern of the recombinantly produced GCS of the present invention is more like that of the human liver, spleen, brain or macrophage GCS, as compared to the placental enzyme and obtained in large quantities by the expression vector of the present invention, replacement therapy of Gaucher's disease now becomes possible for treating patients afflicted with this disease. A method of treating this disease comprises administering to a subject afflicted

with Gaucher's disease, therapeutic amounts of recombinant GCS of the present invention to alleviate said disease condition.

A pharmaceutical composition comprises therapeutic amounts of the GCS of the present invention and pharmaceutically acceptable carrier such as physiological saline, non-toxic sterile buffers and the like.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

WHAT IS CLAIMED IS

1. A cDNA clone containing complete coding region for human lysosomal glycoprotein glucocerebrosidase, said coding region comprising nucleotide sequence as follows:

```
  G GAG TTT TCA AGT CCT TCC AGA GAG GAA TGT CCC AAG CCT TTG AGT
AGG GTA AGC ATC ATG GTC GGC AGC CTC ACA GGA TTG CTT TCA CTT CAG
GCA GTG TCG TGG CGA TCA GGT GCC CGC CCC TGC ATC CCT AAA AGC TTC
GGC TAC AGC TCG GTG GTG TGT GTC TGC AAT GCC ACA TAC TGT GAC TCC
TTT GAC CCC CCG ACC TTT CCT GCC CTT GGT ACC TTC AGC CGC TAT GAG
AGT ACA CGC AGT GGG CGA GCC ATG GAG CTG AGT ATG GGG CCC ATC CAG
GCT AAT CAC ACG GGC ACA GGC CTG CTA CTG ACC CTG CAG CCA GAA CAG
AAG TTC CAG AAA GTG AAG GGA TTT GGA GGG GCC ATG ACA GAT GCT GCT
GCT CTC AAC ATC CTT GCC CTG TCA CCC CCT GCC CAA AAT TTG CTA CTT
AAA TCG TAC TTC TCT GAA GAA GGA ATC GGA TAT AAC ATC ATC CGG GTA
CCC ATG GCC AGC TGT GAC TTC TCC ATC CGC ACC TAC ACC TAT GCA GAC
ACC CCT GAT GAT TTC CAG TTG CAC AAC TTC AGC CTC CCA GAG GAA GAT
ACC AAG CTC AAG ATA CCC CTG ATT CAC CGA GCC CTG CAG TTG GCC CAG
CGT CCC GTT TCA CTC CTT GCC AGC CCC TGG ACA TCA CCC ACT TGG CTC
AAG ACC AAT GGA GCG GTG AAT GGG AAG GGG TCA CTC AAG GGA CAG CCC
GGA GAC ATC TAC CAC CAG ACC TGG GCC AGA TAC TTT GTG AAG TTC CTG
GAT GCC TAT GCT GAG CAC AAG TTA CAG TTC TGG GCA GTG ACA GCT GAA
AAT GAG CCT TCT GCT GGG CTG TTG AGT GGA TAC CCC TTC CAG TGC CTG
GGC TTC ACC CCT GAA CAT CAG CGA GAC TTC ATT GCC CGT GAC CTA GGT
CCT ACC CTC GCC AAC AGT ACT CAC CAC AAT GTC CGC CTA CTC ATG CTG
GAT GAC CAA CGC TTG CTG CTG CCC ACC TGG GCA AAG GTG GTA CTG ACA
GAC CCA GAA GCA GCT AAA TAT GTT CAT GGC ATT GCT GTA CAT TGG TAC
CTG GAC TTT CTG GCT CCA GCC AAA GCC ACC CTA GGG GAG ACA CAC CGC
CTG TTC CCC AAC ACC ATG CTC TTT GCC TCA GAG GCC TGT GTG GGC TCC
AAG TTC TGG GAG CAG AGT GTG CGG CTA GGC TCC TGG GAT CGA GGG ATG
CAG TAC AGC CAC AGC ATC ATC ACG AAC CTC CTG TAC CAT GTG GTC GGC
TGG ACC GAC TGG AAC CTT GCC GTG AAC CCC GAA GGA GGA CCC AAT TGG
GTG CGT AAC TTT GTC GAC AGT CCC ATC ATT GTA GAC ATC ACC AAG GAC
ACG TTT TAC AAA CAG CCC ATG TTC TAC CAC CTT GGC CAC TTC AGC AAG
TTC ATT CCT GAG GGC TCC CAG AGA GTG GGG CTG GTT GCC AGT CAG AAG
AAC GAC CTG GAC GCA GTG GCA TTG ATG CAT CCC GAT GGC TCT GCT GTT
GTG GTC GTG CTA AAC CGC TCC TCT AAG GAT GTG CCT CTT ACC ATC AAG
GAT CCT GCT GTG GGC TTC CTG GAG ACA ATC TCA CCT GGC TAC TCC ATT
CAC ACC TAC CTG TGG CGT CGC CAG TGA TGG AGC AGA TAC TCA AGG AGG
CAC TGG GCT CAG CCT GGG CAT TAA AGG GAC A
```

2. The clone of claim 1 inserted into baculovirus genome.

3. The clone of claim 2 directing synthesis of unique glucocerebrosidase when Spodoptera frugiperda cells are infected therewith.

4. Isolated, substantially pure synthetic glycoprotein glucocerebrosidase wherein carbohydrate structure of said glycoprotein is different from human placental glucocerebrosidase.

5. The glycoprotein having the following amino acid sequence:

Met Ala Gly Ser Leu Thr Gly Leu Leu Leu Leu Gln
Ala Val Ser Trp Ala Ser Gly Ala Arg Pro Cys Ile Pro Lys Ser Phe
Gly Tyr Ser Ser Val Val Cys Val Cys Asn Ala Thr Tyr Cys Asp Ser
Phe Asp Pro Pro Thr Phe Pro Ala Leu Gly Thr Phe Ser Arg Tyr Glu
Ser Thr Arg Ser Gly Arg Arg Met Glu Leu Ser Met Gly Pro Ile Gln
Ala Asn His Thr Gly Thr GLy LEu LEu Leu Thr Leu Gln Pro Glu Gln
Lys Phe Gln Lys Val Lys Gly Phe Gly Gly Ala Met Thr Aps Ala Ala
Ala Leu Asn Ile Leu Ala Leu Ser Pro Pro Ala Gln Asn Leu Leu Leu
Lys Ser Tyr Phe Ser Glu Glu Gly Ile Gly Tyr Ana Ile Ile Arg Val
Pro Met Ala Ser Cys Aps Phe Ser Ile Arg Thr Tyr Thr Tyr Ala Asp
Thr Pro Aps Aps Phe Gln Leu His Asn Phe Ser Leu Pro Glu GLu Asp
Thr Lys Leu Lys Ile Pro Leu Ile His Arg Ala Leu Gln Leu Ala Gln
Arg Pro Val Ser Leu Leu Ala Ser Pro Trp Thr Ser Pro Thr Trp Leu
Lys Thr Asn GLy Ala Val Asn Gly Lys Gly Ser Leu Lys Gly Gln Pro
Gly Asp Ile Tyr His Gln Thr Trp Ala Arg Tyr Phe Val Lys Phe Leu
Asp Ala Tyr Ala Glu His Lys Leu Gln Phe Trp Ala Val Thr Ala Glu
Asn Glu Pro Ser Ala Gly Leu Leu Ser GLy Tyr Pro Phe Gln Cys Leu
Gly Phe Thr Pro Glu His Gln Arg Asp Phe Ile Ala Arg Asp Leu Gly
Pro Thr Leu Ala Asn Ser Thr His His Asn Val Arg Leu Leu Met Leu
Asp Asp Gln Arg Leu Leu Leu Pro His trp Ala Lys Val Val Leu Thr
Asp Pro Glu Ala Ala Lys Try Val His Gly Ile Ala Val His Trp Tyr
Leu Asp Phe Leu Ala Pro Ala Lys Ala Thr Leu Gly Glu Thr His Arg
Leu Phe Pro Asn Thr Met Leu Phe Ala Ser Glu Ala Cys Val Gly Ser
Lys Phe Trp Glu Gln Ser Val Arg Leu Gls Ser Trp Asp Arg Gly Met
Gln Tyr Ser His Ser Ile Ile Thr Asn Leu Leu Tyr His Val Val Gly
Trp Thr Asp Trp Asn Leu Ala Leu Asn Pro Glu Gly Gly Pro Asn Trp
Val Arg Asn Phe Val Asn Ser Pro Ile Ile Val Asp Ile Thr Lys Asp
Thr Phe Tyr Lys Gln Pro Met Phe Tyr His Leu Gly His Phe Ser Lys
Phe Ile Pro Glu Gly Ser Gln Arg Val GLy Leu Val Ala Ser Gln Lys
Asn Asp Leu Asp Ala Val Ala Leu met His Pro Asp Gly Ser Ala Val
Val Val Val Leu Asn Arg Ser Ser Lys Asp Val Pro Leu Thr Ile Lys
Asp Pro Ala Val Gly Phe Leu Glu Thr Ile Ser Pro Gly Tyr Ser Ile
His Thr Tyr Leu Trp Arg Arg Gln

6. A pharmaceutical composition comrising therapeutic amount of the glycoprotein of claim 4, and pharmaceutically acceptable carrier.

7. A method of treating Gaucher's disease, comprising administering to a subject inflicted with Gaucher's disease, therapeutic amount of the glycoprotein of claim 4 to alleviate Gaucher's disease.

8. The clone of claim 1 having the characteristics of ATCC 40393.

```
        G GAG TTT TCA AGT CCT TCC AGA GAG GAA TGT CCC AAG CCT TTG AGT      46

                      Met Ala Gly Ser Leu Thr Gly Leu Leu Leu Leu Gln      12
 47  AGG GTA AGC ATC ATG GCT GGC AGC CTC ACA GGA TTG CTT CTA CTT CAG      94

 13  Ala Val Ser Trp Ala Ser Gly Ala Arg Pro Cys Ile Pro Lys Ser Phe      28
 95  GCA GTG TCG TGG GCA TCA GGT GCC CGC CCC TGC ATC CCT AAA AGC TTC     142

 29  Gly Tyr Ser Ser Val Val Cys Val Cys Asn Ala Thr Tyr Cys Asp Ser      44
143  GGC TAC AGC TCG GTG GTG TGT GTC TGC AAT GCC ACA TAC TGT GAC TCC     190

 45  Phe Asp Pro Pro Thr Phe Pro Ala Leu Gly Thr Phe Ser Arg Tyr Glu      60
191  TTT GAC CCC CCG ACC TTT CCT GCC CTT GGT ACC TTC AGC CGC TAT GAG     238

 61  Ser Thr Arg Ser Gly Arg Arg Met Glu Leu Ser Met Gly Pro Ile Gln      76
239  AGT ACA CGC AGT GGG CGA CGG ATG GAG CTG AGT ATG GGG CCC ATC CAG     286

 77  Ala Asn His Thr Gly Thr Gly Leu Leu Leu Thr Leu Gln Pro Glu Gln      92
287  GCT AAT CAC ACG GGC ACA GGC CTG CTA CTG ACC CTG CAG CCA GAA CAG     334

 93  Lys Phe Gln Lys Val Lys Gly Phe Gly Gly Ala Met Thr Asp Ala Ala     108
335  AAG TTC CAG AAA GTG AAG GGA TTT GGA GGG GCC ATG ACA GAT GCT GCT     382

109  Ala Leu Asn Ile Leu Ala Leu Ser Pro Pro Ala Gln Asn Leu Leu Leu     124
383  GCT CTC AAC ATC CTT GCC CTG TCA CCC CCT GCC CAA AAT TTG CTA CTT     430.

125  Lys Ser Tyr Phe Ser Glu Glu Gly Ile Gly Tyr Asn Ile Ile Arg Val     140
431  AAA TCG TAC TTC TCT GAA GAA GGA ATC GGA TAT AAC ATC ATC CGG GTA     478
```

FIG. 1-1

141	Pro	Met	Ala	Ser	Cys	Asp	Phe	Ser	Ile	Arg	Thr	Tyr	Thr	Tyr	Ala	Asp	156
479	CCC	ATG	GCC	AGC	TGT	GAC	TTC	TCC	ATC	CGC	ACC	TAC	ACC	TAT	GCA	GAC	526
157	Thr	Pro	Asp	Asp	Phe	Gln	Leu	His	Asn	Phe	Ser	Leu	Pro	Glu	Glu	Asp	172
527	ACC	CCT	GAT	GAT	TTC	CAG	TTG	CAC	AAC	TTC	AGC	CTC	CCA	GAG	GAA	GAT	574
173	Thr	Lys	Leu	Lys	Ile	Pro	Leu	Ile	His	Arg	Ala	Leu	Gln	Leu	Ala	Gln	188
575	ACC	AAG	CTC	AAG	ATA	CCC	CTG	ATT	CAC	CGA	GCC	CTG	CAG	TTG	GCC	CAG	622
189	Arg	Pro	Val	Ser	Leu	Leu	Ala	Ser	Pro	Trp	Thr	Ser	Pro	Thr	Trp	Leu	204
623	CGT	CCC	GTT	TCA	CTC	CTT	GCC	AGC	CCC	TGG	ACA	TCA	CCC	ACT	TGG	CTC	670
205	Lys	Thr	Asn	Gly	Ala	Val	Asn	Gly	Lys	Gly	Ser	Leu	Lys	Gly	Gln	Pro	220
671	AAG	ACC	AAT	GGA	GCG	GTG	AAT	GGG	AAG	GGG	TCA	CTC	AAG	GGA	CAG	CCC	718
221	Gly	Asp	Ile	Tyr	His	Gln	Thr	Trp	Ala	Arg	Tyr	Phe	Val	Lys	Phe	Leu	236
719	GGA	GAC	ATC	TAC	CAC	CAG	ACC	TGG	GCC	AGA	TAC	TTT	GTG	AAG	TTC	CTG	766
237	Asp	Ala	Tyr	Ala	Glu	His	Lys	Leu	Gln	Phe	Trp	Ala	Val	Thr	Ala	Glu	252
767	GAT	GCC	TAT	GCT	GAG	CAC	AAG	TTA	CAG	TTC	TGG	GCA	GTG	ACA	GCT	GAA	814
253	Asn	Glu	Pro	Ser	Ala	Gly	Leu	Leu	Ser	Gly	Tyr	Pro	Phe	Gln	Cys	Leu	268
815	AAT	GAG	CCT	TCT	GCT	GGG	CTG	TTG	AGT	GGA	TAC	CCC	TTC	CAG	TGC	CTG	862
269	Gly	Phe	Thr	Pro	Glu	His	Gln	Arg	Asp	Phe	Ile	Ala	Arg	Asp	Leu	Gly	284
863	GGC	TTC	ACC	CCT	GAA	CAT	CAG	CGA	GAC	TTC	ATT	GCC	CGT	GAC	CTA	GGT	910
285	Pro	Thr	Leu	Ala	Asn	Ser	Thr	His	His	Asn	Val	Arg	Leu	Leu	Met	Leu	300
911	CCT	ACC	CTC	GCC	AAC	AGT	ACT	CAC	CAC	AAT	GTC	CGC	CTA	CTC	ATG	CTG	958

FIG. 1-2

301	Asp	Asp	Gln	Arg	Leu	Leu	Leu	Pro	His	Trp	Ala	Lys	Val	Val	Leu	Thr	316
959	GAT	GAC	CAA	CGC	TTG	CTG	CTG	CCC	CAC	TGG	GCA	AAG	GTG	GTA	CTG	ACA	1006
317	Asp	Pro	Glu	Ala	Ala	Lys	Tyr	Val	His	Gly	Ile	Ala	Val	His	Trp	Tyr	332
1007	GAC	CCA	GAA	GCA	GCT	AAA	TAT	GTT	CAT	GGC	ATT	GCT	GTA	CAT	TGG	TAC	1054
333	Leu	Asp	Phe	Leu	Ala	Pro	Ala	Lys	Ala	Thr	Leu	Gly	Glu	Thr	His	Arg	348
1055	CTG	GAC	TTT	CTG	GCT	CCA	GCC	AAA	GCC	ACC	CTA	GGG	GAG	ACA	CAC	CGC	1102
349	Leu	Phe	Pro	Asn	Thr	Met	Leu	Phe	Ala	Ser	Glu	Ala	Cys	Val	Gly	Ser	364
1103	CTG	TTC	CCC	AAC	ACC	ATG	CTC	TTT	GCC	TCA	GAG	GCC	TGT	GTG	GGC	TCC	1150
365	Lys	Phe	Trp	Glu	Gln	Ser	Val	Arg	Leu	Gly	Ser	Trp	Asp	Arg	Gly	Met	380
1151	AAG	TTC	TGG	GAG	CAG	AGT	GTG	CGG	CTA	GGC	TCC	TGG	GAT	CGA	GGG	ATG	1198
381	Gln	Tyr	Ser	His	Ser	Ile	Ile	Thr	Asn	Leu	Leu	Tyr	His	Val	Val	Gly	396
1199	CAG	TAC	AGC	CAC	AGC	ATC	ATC	ACG	AAC	CTC	CTG	TAC	CAT	GTG	GTC	GGC	1246
397	Trp	Thr	Asp	Trp	Asn	Leu	Ala	Leu	Asn	Pro	Glu	Gly	Gly	Pro	Asn	Trp	412
1247	TGG	ACC	GAC	TGG	AAC	CTT	GCC	CTG	AAC	CCC	GAA	GGA	GGA	CCC	AAT	TGG	1294
413	Val	Arg	Asn	Phe	Val	Asp	Ser	Pro	Ile	Ile	Val	Asp	Ile	Thr	Lys	Asp	428
1295	GTG	CGT	AAC	TTT	GTC	GAC	AGT	CCC	ATC	ATT	GTA	GAC	ATC	ACC	AAG	GAC	1342

FIG. 1-3

```
 429  Thr Phe Tyr Lys Gln Pro Met Phe Tyr His Leu Gly His Phe Ser Lys   444
1343  ACG TTT TAC AAA CAG CCC ATG TTC TAC CAC CTT GGC CAC TTC AGC AAG  1390

 445  Phe Ile Pro Glu Gly Ser Gln Arg Val Gly Leu Val Ala Ser Gln Lys   460
1391  TTC ATT CCT GAG GGC TCC CAG AGA GTG GGG CTG GTT GCC AGT CAG AAG  1438

 461  Asn Asp Leu Asp Ala Val Ala Leu Met His Pro Asp Gly Ser Ala Val   476
1439  AAC GAC CTG GAC GCA GTG GCA TTG ATG CAT CCC GAT GGC TCT GCT GTT  1486

 477  Val Val Val Leu Asn Arg Ser Ser Lys Asp Val Pro Leu Thr Ile Lys   492
1487  GTG GTC GTG CTA AAC CGC TCC TCT AAG GAT GTG CCT CTT ACC ATC AAG  1534

 493  Asp Pro Ala Val Gly Phe Leu Glu Thr Ile Ser Pro Gly Tyr Ser Ile   508
1535  GAT CCT GCT GTG GGC TTC CTG GAG ACA ATC TCA CCT GGC TAC TCC ATT  1582

 509  His Thr Tyr Leu Trp Arg Arg Gln
1583  CAC ACC TAC CTG TGG CGT CGC CAG TGA TGG AGC AGA TAC TCA AGG AGG  1630


1631  CAC TGG GCT CAG CCT GGG CAT TAA AGG GAC A
```

FIG. 1-4

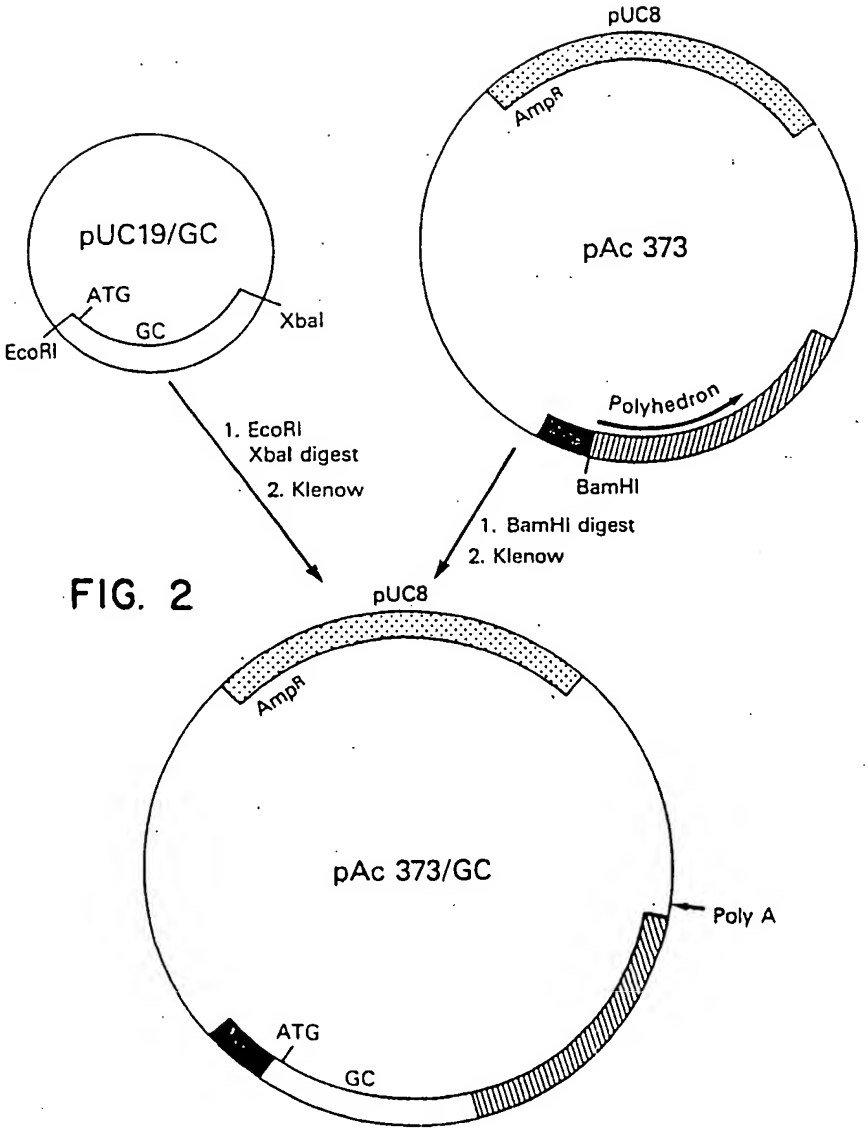
pUC8
AmpR
pUC19/GC
ATG
GC
EcoRI
XbaI
pAc 373
Polyhedron
BamHI
1. EcoRI
XbaI digest
2. Klenow
1. BamHI digest
2. Klenow
pUC8
AmpR
pAc 373/GC
Poly A
ATG
GC

FIG. 2

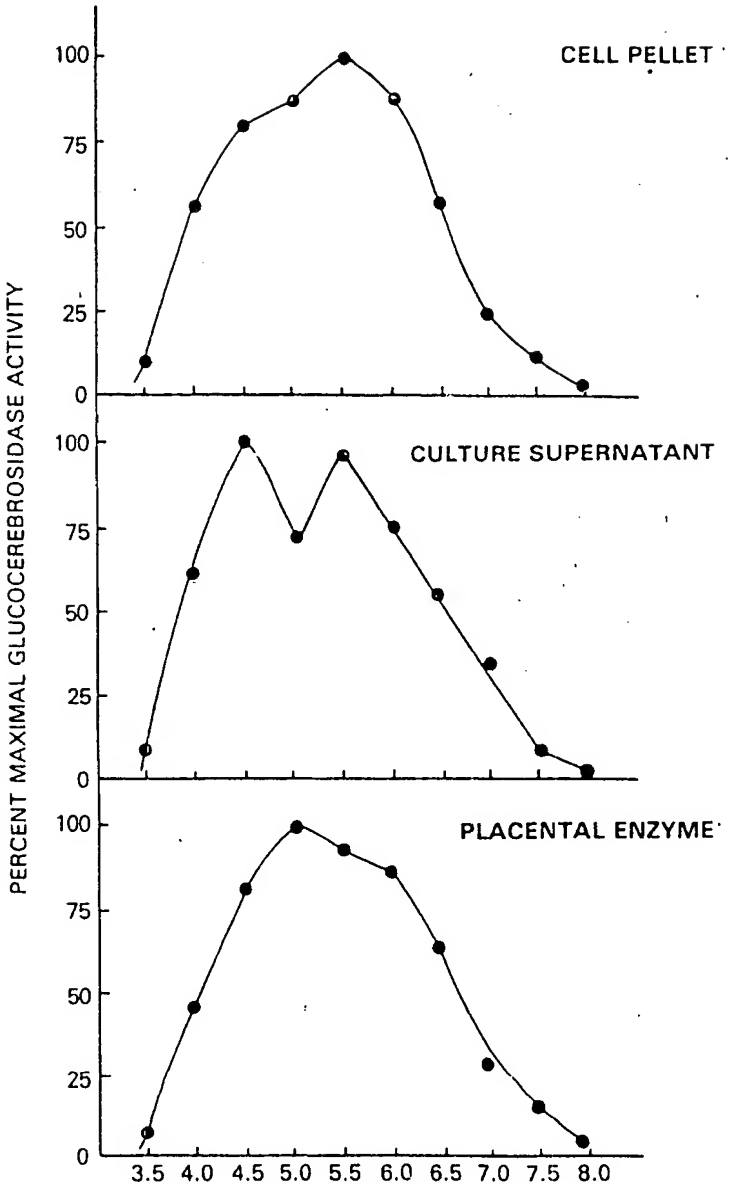
CELL PELLET
CULTURE SUPERNATANT
PLACENTAL ENZYME
PERCENT MAXIMAL GLUCOCEREBROSIDASE ACTIVITY
100
75
50
25
0
3.5 4.0 4.5 5.0 5.5 6.0 6.5 7.0 7.5 8.0
pH

FIG. 3

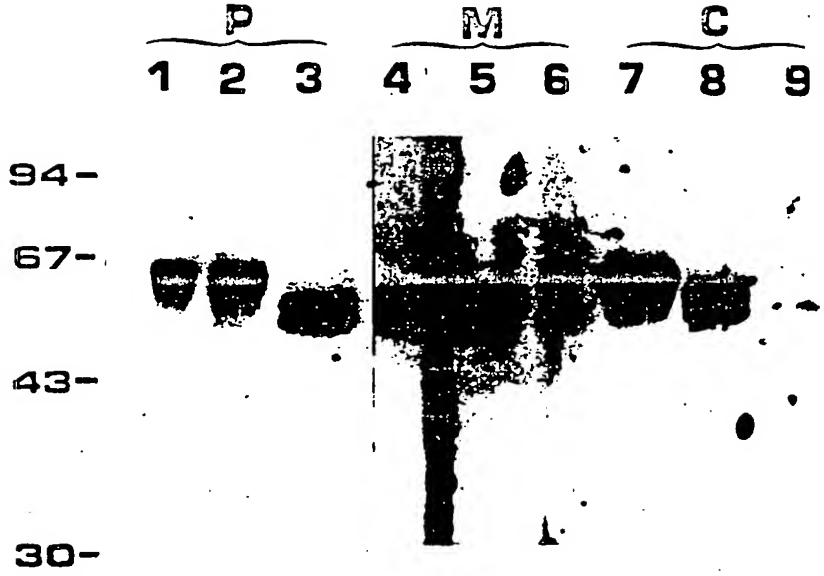

FIG. 4

NTERNATIONAL SEARCH REPC

International Application No. PCT/US88/04314

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (4): C12N 7/00, 9/24, 5/00, 15/00; C07H 21/00; A61K 37/54
U.S. Cl.: 536/27; 435/172.3, 200, 235, 240.2 424/94.61

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	536/27; 435/68, 91, 172.3, 200, 235, 240.2, 320; 424/94.61; 935/32, 57, 70

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

Chemical Abstract Data Base (CAS) 1967-1989. Keywords: Baculovirus, Glucocerebrosidase, Vector, Gaucher, Recombin?

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Proceedings of the National Academy of Sciences USA, Vol 82, issued November, 1985 (Washington, D.C.) by J. Sorge et al., "Molecular cloning and nucleotide sequence of human glucocerebrosidase cDNA", see pages 7289-7293. See particularly page 7291.	1, 5 and 8
Y	The Journal of Biological Chemistry, Volume 261, No. 1, issued January, 1986 (Baltimore, Maryland) by S. Tsuji et al, "Nucleotide Sequence of cDNA containing the complete coding sequence for human lysosomal Glucocerebrosidase", see pages 50-53. See particularly page 52.	1, 5 and 8
Y, P	US, A, 4,745,051 (SMITH) 17 May 1988. See entire document.	2 and 3

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 February 1989	29 MAR 1989
International Searching Authority	Signature of Authorized Officer
ISA/US	Mary E. Pratt Mary E. Pratt

Form PCT/ISA/210 (second sheet) (Rev. 11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Journal of Biological Chemistry, Volume 260, No. 26, issued 15 November 1985 (Baltimore, Maryland) A. H. Erickson et al., "Biosynthesis of the Lysosomal Enzyme Glucocerebrosidase." See pages 14319-14324. See particularly 14321 and 14323.	4
Y		5,6, and 7
Y	Journal of General Vivology, Volume 68, Part 5, issued May 1987 (Great Britain) Y Matsuura et al., "Baculovirus Expression vectors: the requirements for High Level Expression of Proteins, Including Glycoproteins, "see entire document, pages 1233-1250.	2, 3, and 8
A	Journal of Virology, Volume 60, No. 3, issued March 1987 (Washington, D.C.), K.-T. Jeang et al, "Abundant Synthesis of Functional Human T-Cell Leukemia Virus Type I $p40^{X}$ Protein in Eukaryotic Cells by using a Baculovirus Expression Vector", See pages 708-710.	2, 3, and 8
A	Proceedings of the National Academy of Sciences, USA, Volume 74, No. 8, issued August 1977 (Washington, D.C.) F.S. Furbish et al., "Enzyme replacement therapy in Gaucher's disease: Large-scale purification of glucocerebrosidase suitable for human administration", see p. 3560-3563.	6 and 7